SUPPLEMENTAL MATERIALS

Antibodies and Reagents - FK2 (mAb mono and poly-ubiquitin conjugate) antibody was purchased from Biomol (Plymouth Meeting, PA). Anti-synapsin antibody (pAb) was purchased from Chemicon (Temecula, CA).

Drug treatment and imaging of fixed cells – Mature hippocampal neuron cultures (>17 DIV) were treated with various drugs or vehicle (DMSO) in conditioned growth media. Cells were then fixed in 4% paraformaldahdye/4% sucrose for 10 minutes, and blocked and permeabilized in PBS-MC (phosphate buffered saline with 1 mM MgCl2 and 0.1 CaCl2) with Triton X-100 (0.2%) and BSA (2%). Cells were incubated overnight with anti-ubiquitin conjugate specific (FK2 mAb, 1:1000) and anti-synapsin (pAb, 1:1000) antibodies diluted in PBS-MC plus 2% BSA. Cells were washed and incubated with goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 568 secondary antibodies (1:1000) diluted in PBS-MC plus 2% BSA. Cells were then washed and coverslips were mounted in Aqua Poly/Mount (Polysciences, Warrington, PA). For proteasome perturbation experiments in Supplementary Figure 1, cultures were treated with MG132 (5μM), vinyl sulfone (1 μM), epoxomicin (1 μM) or calpeptin (10 μM). For quantitation of FK2 (ubiquitin conjugate) immunofluorescence, max projected confocal z-stacks were analyzed with NIH ImageJ. FK2 images were thresholded to a level at least 1.5-2x times background (minimum gray values). The mean FK2 fluorescence intensity integrated from 63X fields of neurons is plotted relative to control treated neurons. Three separate experiments were performed for each condition, with each experiment normalized to its own control.

Evaluation of CaMKIIα on 26S proteasome activity - The effect of CaMKIIα on 26S proteasome activity was evaluated by measuring rates of Suc-LLVY-AMC hydrolysis. 26S proteasome was purified from bovine red cells as described previously (1). 26S proteasome was pre-incubated in the presence or absence of activated CaMKIIα as described for 19S. 10 nM 26S proteasome samples were assayed for peptidase activity as described previously (2).

Evaluation of CaMKIIα on 19S-dependent activation of 20S proteasome activity - The effect of CaMKIIα on 19S-dependent activation of 20S proteasome activity was evaluated as described previously (2,3). Purified 19S was pre-incubated in the presence or absence of activated CaMKIIα prior to incubation with 20S proteasome and assay of peptidase activity.

Mass spectrometry. Protein identification by mass spectrometry was conducted by the Protein Chemistry Core Research Facility at UT Southwestern Medical Center.

SUPPLEMENTARY FIGURE LEGENDS

Supplemental Figure 1. Proteasome inhibition or action potential blockade rapidly increase ubiquitin conjugate levels in hippocampal neurons. *A*, To determine the specificity of our ubiquitin conjugate-specific antibody (FK2) in situ method, we treated hippocampal cultures with either control, MG132 (5 uM), vinyl sulfone (1 uM), epoxomicin (1 uM) or calpeptin (10 uM) for 10 minutes. Straightened dendrites (with cell body included) of representative max z-projected confocal images are shown. As depicted proteasome inhibitors increase ubiquitin conjugate levels (FK2), while the calpain specific inhibitor, calpeptin, had no effect. Scale bar = 5 microns. *B*, Poly-ubiquitin conjugates from control and MG132 treated neurons are precipitated by GST-S5A, resolved on SDS-PAGE, and western blotted with FK2 antibody. *C*, Treatment of hippocampal neurons with MG132 (10 minutes) induces ubiquitin conjugate (FK2) positive puncta, many of which are juxtaposed with the pre-synaptic marker

synapsin (black arrows; FK2 - green, SYN - red). Scale bar is 10 microns. *D,* Action potential (AP) blockade with TTX rapidly increases ubiquitin conjugate levels in hippocampal neurons. Neurons (21 DIV) were immunostained with FK2 after treatment with vehicle (DMSO) or TTX (2 μM) for 10 minutes. Dendrites from each treatment are depicted. Scale bar = 5 microns. *E,* Co-application of the proteasome inhibitor MG132 with TTX for 10 minutes uncovers activity-regulated proteasome inhibition in hippocampal neurons. Relative to control treated neurons, TTX or MG132 rapidly increased ubiquitin conjugate levels at 10 minutes. The levels of ubiquitin conjugates induced by co-application of TTX and MG132 were not significantly different from either alone (*, $P < 0.001$). This suggests that action potential blockade with TTX mimics proteasome inhibition. Analysis of total FK2 fluorescence is depicted in bar graph (mean ± SEM). Three separate experiments performed. $n = 30$ individual 63x fields (2-3 cells per field)).

Supplemental Figure 2. CaMKII T286D increases proteasome activity in HEK293T cells. *A,* Representative images of HEK293T cells co-transfected with GFPu degradation reporter and CaMKII T286D or empty vector (Control) for 20 hours, and then treated with MG132 (25 uM) for 2 hours. (Scale bar = 25 micron; Color lookup is black to white, low to high fluorescence; n=3). *B,* Bar graph comparing total GFPu fluorescence in HEK293T cells after 20 hours. The steady state level of GFPu fluorescence is significantly lower in HEK293T cells transfected with CaMKII T286D (*, $p < 0.01$). *C,* Bar graph comparing total GFPu fluorescence of untreated and MG132 treated HEK293T cells. Incubation with MG132 for 2 hours increases GFPu fluorescence in both control and CaMKII T286D transfected cells.

Supplemental Figure 3. Phosphorylation of 19S by CaMKII does not regulate proteasome function *in vitro*. *A,* CaMKIIα rapidly phosphoylated 19S proteasome. Purified CaMKIIα was added to purified MBP (CaMKIIα control substrate) or 19S complex for up to 180 minutes *in vitro*. Autoradiography shows 19S proteasome subunits of ~45-47 kDa (black arrow) ^{32}P labeled by CaMKIIα within 15 minutes (top). Total protein stain by Coomassie (bottom) displays all 19S proteasome subunits. *B,* Purified CaMKIIα was added to purified 26S proteasome and subsequently assayed for Suc-LLVY-AMC hydrolysis. Phosphorylation by CaMKIIα had no effect on proteasome activity. No increase in proteasome activity (as measured by AMC fluorescence) over control (no kinase) was observed. *C,* Purified 20S (core) was added to purified 19S (cap) proteasome with or without addition of purified CaMKIIα and subsequently assayed for Suc-LLVY-AMC hydrolysis. Phosphorylation by CaMKIIα had no effect on proteasome core-cap assembly. An increase in assembly would lead to an increase in proteasome activity. No increase in AMC fluorescence was observed.

Supplemental Figure 4. CaMKII dependent phosphorylation of proteasome or proteasome interacting proteins *in vivo*. HeK293T cells were transfected with either empty vector or constitutively active CaMKII (T286D) and labeled with ^{32}P orthophosphate. *A,* Representative autoradiogram for ^{32}P orthophosphate labeling of immunoprecipitated 26S proteasome (IP: α2 proteasome). White arrow denotes CaMKII stimulated phosphorylation. n=3. *B,* Line scan analysis of autoradiogram from (A) highlights the induction of a phosphorylated protein at around 45-47 kDa (black arrow) similar to the molecular weight of Rpt6. *C,* Western Blot analysis of immunoprecipitated core (αβ prot) or 19S (Rpt6) proteasomes.

Supplemental Movie 1. paGFPu degradation in BIC treated hippocampal neurons. This movie is a time-lapse image sequence of paGFPu degradation in a segment of a representative dendrite from a paGFPu expressing neuron treated with BIC (40 μM). BIC was added after 10 minutes of baseline imaging. Confocal time-lapse images were acquired at 63x every 2 minutes.

The time-lapse image sequence was processed to highlight the rates in fluorescence decay between successive frames (F_n - F_{n-1}/F_{n-1}). The color look-up scale for paGFPu degradation is black (lowest) to red (highest). As depicted, BIC rapidly induces paGFPu degradation that was initiated and persisted in spines. Scale bar is 5 μm.

REFERENCES

1. Liu, C. W., Li, X., Thompson, D., Wooding, K., Chang, T. L., Tang, Z., Yu, H., Thomas, P. J., and DeMartino, G. N. (2006) *Mol Cell* 24(1), 39-50
2. DeMartino, G. N., Moomaw, C. R., Zagnitko, O. P., Proske, R. J., Ma, C-P, Afendis, S. J., Swaffield, J. C., and Slaughter, C. A. (1994) *J Biol Chem* 269(33), 20878-20884
3. Ma, C-P, Vu, J. H., Proske, R. J., Slaughter, C. A., and DeMartino, G. N. (1994) *J Biol Chem* 269(5), 3539-3547

Supplemental Table 1. Djakovic et al.

Conditions	*Rate of Degradation*[1]	*p-value*	*Number of imaged dendrites*
Control[a]	1.09 +/- 0.17		14
MG132	-0.11 +/- 0.18	< 0.001	14
Calcium Free	0.22 +/- 0.23	0.007	16
TTX	0.13 +/- 0.10	< 0.001	12
BIC	1.62 +/- 0.17	0.036	15
KN-93	0.88 +/- 0.04	0.274	12
BIC[a]	1.62 +/- 0.17		15
KN-93 + BIC	1.03 +/- 0.16	0.021	12
BIC, t:8 min[a]	7.40 +/- 0.77		15
Control, t:8 min	1.53 +/- 0.45	< 0.001	14
KN-93 + BIC, t:8 min	1.46 +/- 0.34	< 0.001	12

[1]Percent fluorescence decay per 4 minute interval. [a]Indicates Reference Category. BIC = Bicuculline. p-value determined by two-tailed unpaired student's t-test.

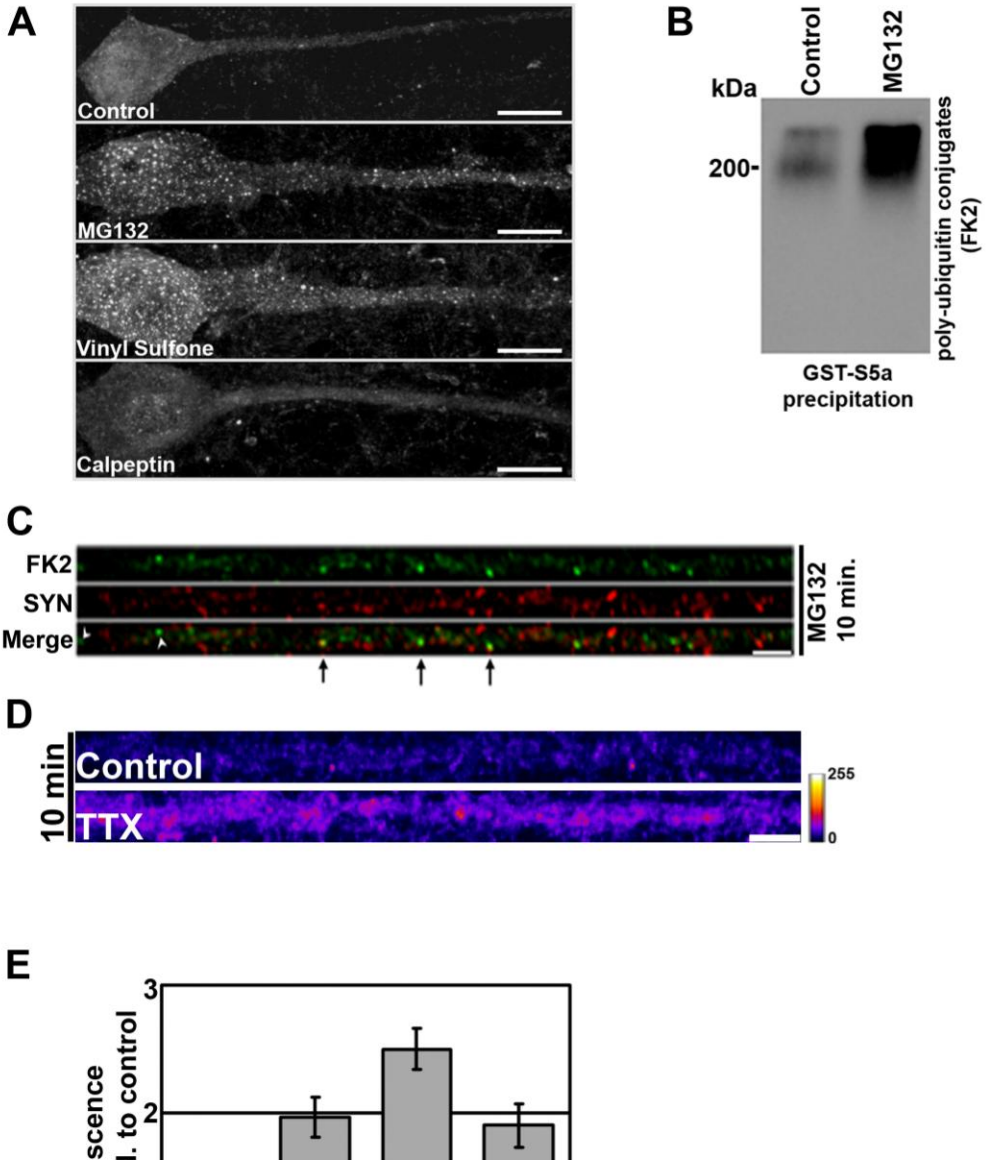
Supplemental Table 1. paGFP-odc mean rates of degradation. Rates are listed as percent fluorescent decay (± SEM) per 2 or 4 minute interval respectively. *P*-values listed are in comparison to the reference group using two-tailed unpaired students T-test. n = number of imaged dendrites.

Supplemental Table 2. Djakovic et al.

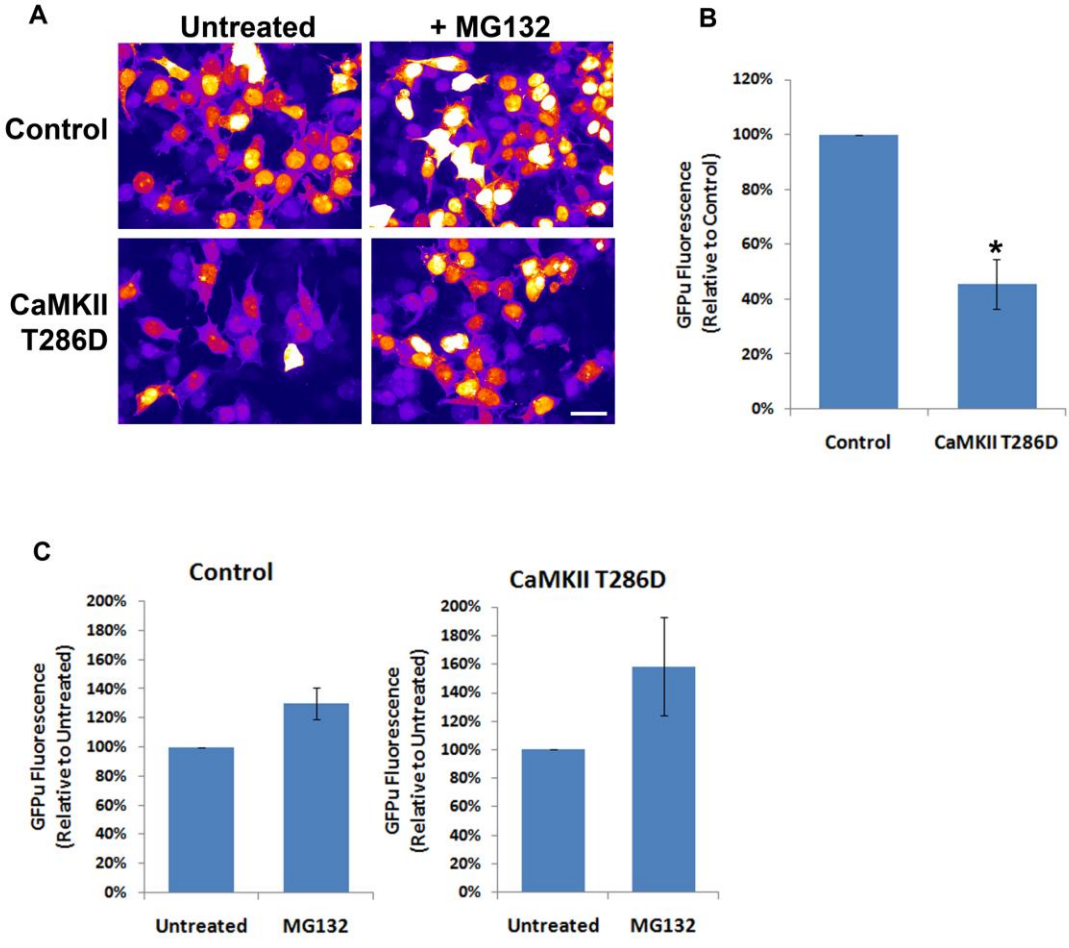
Rpt6 tryptic peptides
R.VSGSELVQK
K.VIMATNR.I
K.GVLLYGPPGTGK
K.IEFPPPNEEAR
K.IAELMPGASGAEVK
R.VHVTQEDFEMAVAK
R.NDSYTLHK
R.HPELFEALGIAQPK
K.VDPLVSLMMVEK.V
R.LEGGSGGDSEVQR.T
R.TMLELLNQLDGFEATK.N
R.EHAPSIIFMDEIDSIGSSR.L
K.EVIELPVKHPELFEALGIAQPK.G

Supplemental Table 2. Identification of Rpt6 by mass spectrometry. Purified 19S/PA700 was incubated with CaMKII for phosphorylation with ^{32}P-ATP. The area of the gel containing the labeled subunit was excised; the protein was solubilized and rerun on SDS-PAGE. The area of label protein was excised, subjected to treatment with trypsin for identification of peptides by mass spectrometry. Only peptides corresponding to 19S subunit Rpt6 were identified (18.5% coverage).

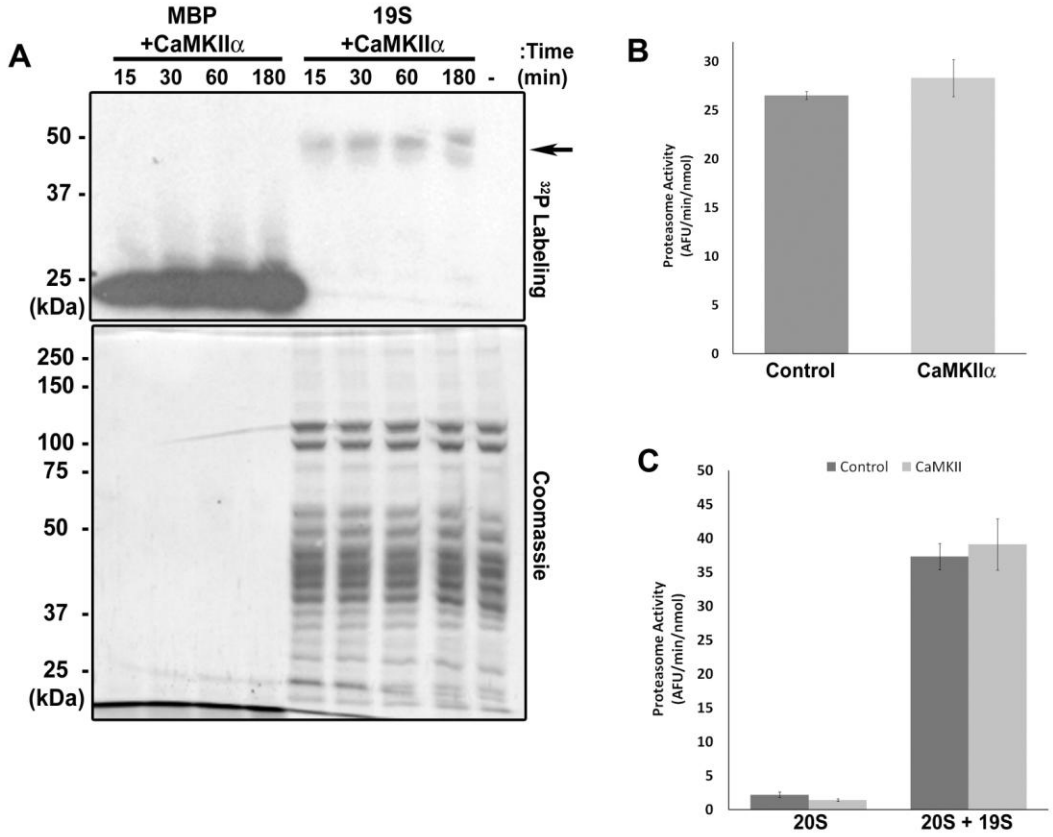
Supplemental Figure 1 - Djakovic et al.



Supplemental Figure 2 - Djakovic et al.



Supplemental Figure 3 - Djakovic et al.



Supplemental Figure 4 - Djakovic et al.